Circulating AMH, antral follicle count and ovulation rate after unilateral ovariectomy in cattle: influence of the bovine fecundity allele Trio

Honors Research Thesis

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ABSTRACT

Trio, a novel high ovulation allele in beef cattle, reduces ovarian follicular growth rate and results in multiple smaller sized ovulatory follicles in contrast to the single ovulatory follicle in wild-type cattle. The objective was to evaluate the effect of unilateral ovariectomy (ULO, surgical removal of one ovary) on antral follicle count (AFC; > 1mm) and anti-Müllerian Hormone (AMH) in carriers and non-carriers of the Trio allele (n = 9/group). On D0, the ovary containing the largest follicle was surgically removed by flank laparotomy. Serum AMH, AFC, and ovulation rate were determined before ULO and at multiple times after ULO until D230. Data were analyzed using linear mixed models and generalized estimating equations. Prior to ULO, AMH and AFC were not different ($P > 0.7$) for Trio carriers (349.7 ± 51 pg/ml; 21.2 ± 1.9 follicles) and non-carriers (334.8 ± 49 pg/ml; 22.1 ± 1.4 follicles). Ovulation rate was greater ($P < 0.01$) in Trio carriers (3.2 ± 0.1) than non-carriers (1.2 ± 0.1) both prior and after ULO, indicating that ULO did not affect ovulation rate. On D1, AFC decreased to 49.7% (carriers) and 50.6% (non-carriers) of pre-ULO values. AFC returned to control values (100%) in 77.8% of heifers, but timing of return was earlier in Trio carriers (65.8 ± 14.3 d) than non-carriers (131.0 ± 29 d). By D230, AFC was similar to pre-ULO in Trio carriers (119%) and non-carriers (91%). For AMH, there was an effect ($P < 0.01$) of genotype, day, and genotype by day interaction on percentage change in circulating AMH. By D3, circulating AMH decreased in both Trio carriers (57.4%) and non-carriers (48.8%). By D230, AMH returned to pre-ULO values in Trio carriers (94.6%) but not in non-carriers (48.1%). Unique observations were: 1) ULO decreased AFC and AMH to ~50%, 2) AFC eventually returned to pre-ULO values by 2 (Trio carriers) to 4 (non-carriers) months, and 3) ovarian compensation, based on AMH, occurred in Trio carriers (~8 months) but not in non-carriers by conclusion of the study (D230). Results obtained from this study further the understanding of the process of folliculogenesis and the control of ovulation rate in monovular species and can aid in the development of improved strategies to prevent twinning, an undesirable trait in cattle.

INTRODUCTION

The process by which ovarian follicles develop through a series of steps to become ovulatory follicles is termed folliculogenesis. It begins with the activation of primordial follicles (< 40 μm) from a fixed pool of follicles present in each individual at the time of birth. Each follicle contains the female gamete, the oocyte. Activated follicles develop through a series of stages, primary and secondary, until reaching the small antral follicle stage (> 1 mm). An essential step in follicle growth is the proliferation of granulosa cells that surround the oocyte from, a single layer of cells in primordial follicles to multiple layers in secondary and antral follicles. From the antral follicle pool, a cohort of follicles are recruited into a follicular wave of growth from which a dominant follicle will be selected to reach ovulatory status. The length of folliculogenesis, defined as the time required from primordial follicle activation to the preovulatory state, is currently unknown and has been the subject of considerable debate by reproductive biologists.

Unilateral ovariectomy (ULO) results in compensatory ovarian hypertrophy in swine, a polytocous species, as indicated by an increase in follicular fluid weight in the remaining ovary, compared to pre-ULO weight, by day 13 to 19 after ULO [1]. There are, however, inconsistent results in the literature concerning compensatory ovarian hypertrophy in ruminants, particularly cattle. Lammoglia et al. [3] reported that ovarian compensatory hypertrophy does occur in cattle based on the observation that the number of follicles entering a follicular wave was similar between animals with one or both ovaries. Conversely, size of the largest follicle within a follicular wave was less after ULO than pre-ULO. Furthermore, the number of follicular waves and estrous cycle length both remain unchanged after ULO [3]. Results of another study in prepuberal heifers indicated a transient increase in FSH following ULO and an increase in ovarian weight due to increased follicular fluid a week after ULO [4]. Conversely, results from other studies in cattle have failed to identify a compensatory effect, based on determination of follicle numbers, for up to 6 days after ULO [5 - 7]. In addition, results of a study conducted in sheep using transrectal ultrasonography indicated there was no difference in the number of small (1 - 3 mm) and medium (4 mm) sized follicles when expressed on a per ovary basis for up to 2 months following ULO, indicating ovarian compensatory hypertrophy did not occur [2]. Thus, there is need to further investigate the potential presence of an ovarian compensatory mechanism after ULO in cattle. In addition, if ovarian compensation were to occur, it would likely require increased activation of primordial follicles to increase the number of growing follicles. As a result, if carefully monitored,

the interval between ULO and ovarian compensation could provide for an estimation of the true length of folliculogenesis.

The use of the ULO model in cattle has also provided some interesting information regarding changes in ovulation rate (number of ovulations) compared to pre-ULO ovulation rates. Cattle are a monovular species, meaning there is ovulation of a single follicle during each estrous cycle [8]. Typically, cattle present 2 - 3 waves of follicular development per estrous cycle, and each wave consists of the synchronized growth of small antral follicles that can range from 5 to 50 follicles, despite only one will acquire the capacity to ovulate. Thus, follicle selection is the process by which a single follicle from a cohort of small antral follicles develops the capacity to become the ovulatory follicle. This process appears to be independent of the number of small antral follicles that are recruited into each wave of follicular growth [8]. Results from previous studies in cattle using the ULO model indicated there was an increase in the number of ovulations during the first two estrous cycles after the procedure, through poorly defined mechanisms [4, 10]. Conversely, results from other studies have not observed any changes in ovulation rate after ULO [11]. Thus, it remains unclear if ULO has a consistent effect on ovulation rate and the mechanisms underlying it.

The evaluation of sheep with relatively greater than typical ovulation rate phenotypes, such as the Booroola/FecB, has led to the identification of specific genes and pathways with important roles in the control of folliculogenesis [12]. Two factors from the TGF-β superfamily, bone morphogenetic protein 15 (BMP15), and growth differentiation factor-9 (GDF9), are produced by the oocyte and regulate granulosa cell proliferation [12]. Similarly, a high fecundity (ability to produce an abundance of offspring) genotype, called Trio, was recently identified in cattle [13]. Animals that are heterozygous for the Trio allele have an ovulation rate on average of 3.5 ± 0.2, whereas animals without the Trio allele have an ovulation rate of 1.1 ± 0.1 with ~90% having single ovulations [9]. The number of follicular waves and the number of follicles (antral follicle count; AFC) at follicular wave emergence are similar between cattle with and without the Trio allele [9, 14]. In a previous study, AFC and anti-Müllerian hormone (AMH), a marker for follicle numbers, did not differ between cattle carrying the Trio allele and those without the allele, indicating that differences in number of follicles recruited into a wave are not responsible for the greater ovulation rate observed in Trio carriers [9]. Conversely, if the Trio allele is present there is an increase in the relative abundance of SMAD6 mRNA transcript, an inhibitor of BMP15 and

GDF9 which are regulators of granulosa cell proliferation [12, 15]. The increase inhibition of BMP15 and GDF9, observed in animals with the Trio allele, leads to reduced follicle growth rate resulting in smaller preovulatory follicles in cattle with the Trio allele compared to controls (8.9 mm vs 14.9 mm respectively) [16, 17]. Because animals with the Trio allele have smaller follicles, the first selected dominant follicle is not large enough to produce enough endocrine inhibition (inhibin and estrogen) to prevent other follicles from being selected for further development, thus leading to the ovulation of 3 or 4 follicles [8]. Little, however, is known about the effects of SMAD6 during the earlier stages of folliculogenesis. Results from research focused on GDF9 and BMP15 in sheep indicate that TGF-β factors also have an important role in the early stages of folliculogenesis, as indicated by fewer granulosa cells per follicle and smaller sized preantral follicles [18, 19].

Because sheep and cattle with relative greater ovulation phenotypes have a lesser follicular growth rate, and the involvement of TGF-β signaling in early folliculogenesis, it is plausible that the slower development of follicles would result in a delay in the compensatory effect on ovarian follicular development after ULO if such compensation were to be confirmed in cattle. The vast majority of studies in cattle evaluating the effect of ULO have been conducted before the introduction of transrectal ultrasonography and the discovery of AMH and its relationship with folliculogenesis. Anti-Müllerian hormone is a glycoprotein of the transforming growth factor beta (TGF-β) family that is only produced in the gonads of males and females. In females, AMH is produced by the granulosa cells of growing preantral and small antral follicles [20, 21]. The relative amount of AMH produced by granulosa cells depends on the stage of follicular development and the concentrations of AMH is less in larger (10 mm) antral and atretic (regressing) follicles [20, 21]. In cattle, AMH concentration is positively correlated with antral follicle count (AFC) and thus can be used as a tool to measure the dynamic follicle population [20 - 22]. Thus, new developments in our ability to monitor folliculogenesis such as ultrasonography and determination of AMH, provide us with better methods to assess whether ovarian compensation occurs after ULO in cattle.

The unique combination of transrectal ultrasonography to evaluate the antral follicle population and circulating AMH concentration can provide for the opportunity to carefully identify the presence and time required for ovarian compensation to occur after ULO in cattle. Furthermore, previous studies have focused on the short-term ovarian effects of ULO, thus the lack of observed

differences could be the result of insufficient time allowed to detect the presence of a compensatory effect on ovarian follicular development. Finally, the utilization of cattle with the Trio allele can help further our understanding of the role of TGF-β signaling during folliculogenesis. Thus, the objectives of the present study were to determine: 1) the presence of ovarian compensatory hypertrophy after ULO in cattle; 2) the time required for ovarian compensation to occur (i.e. length of folliculogenesis) and the effect of the Trio allele mutation on the interval between ULO and ovarian compensation; and 3) the effects of ULO on ovulation rate in both Trio carrier and non-carrier control heifers. We hypothesized that a compensatory mechanism exists after ULO and would be evidenced by: 1) an increase in the number of antral follicles present in the retained ovary to similar numbers as those observed for both ovaries combined prior to ULO, and 2) an increase in circulating AMH to a concentration equivalent to that prior to ULO. In addition, we hypothesize that the length required for the intact ovary to undergo compensation in follicular development to pre-ovariectomy numbers would be longer in animals with the Trio allele due to the slower follicular growth rate. Finally, we hypothesize that ovulation rate after ULO, however, would remain unchanged in animals of each genotype due to the continuous presence of the follicle selection mechanism.

MATERIALS AND METHODS

Animals and treatments

Heifers with and without the Trio allele (n = 9/per group) were subjected to ULO after follicular wave synchronization as shown in Figure 1. Briefly, two doses of prostaglandin F2α (PGF) were administered IM 24 h apart (D-7 and D-6). Transvaginal ultrasound-guided follicle ablation was performed on D-6 and D-5, as previously described [16], to synchronize the emergence of a new wave of ovarian follicular development. On D-5, a controlled internal drug releasing (CIDR, Zoetis) progesterone device was inserted and left in place for 5 days. The ovary containing the dominant follicle (i.e. largest follicle) was surgically removed by flank laparotomy, under regional anesthesia, at the time of CIDR removal (D0). Twenty-four hours after ULO, a new CIDR was administered and left in place for 5 days.

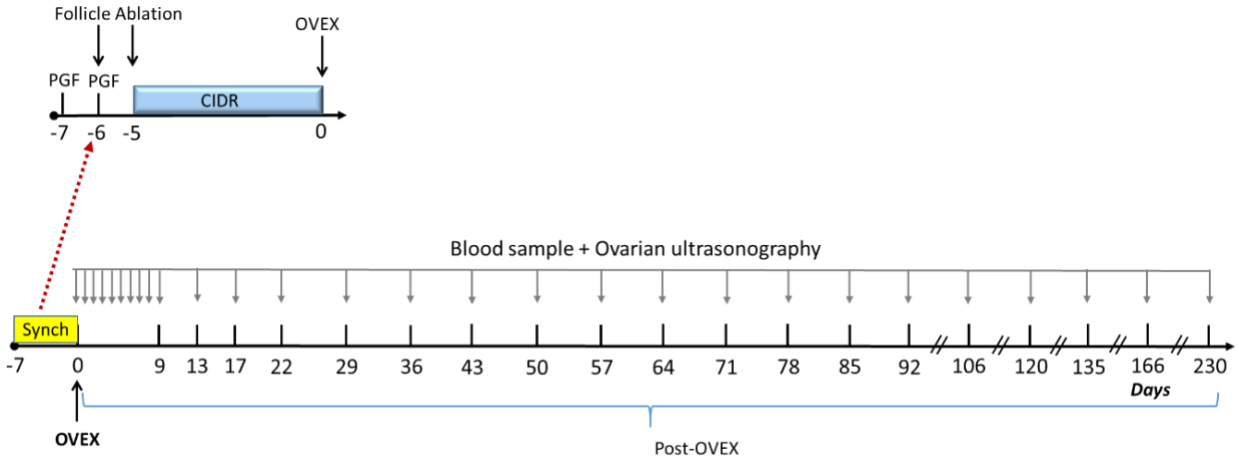

Figure 1. Schedule for treatment and sample collection in both Trio carriers and non-carrier control heifers. Two doses of PGF were administered IM 24 hours apart on D-7 and D-6. Follicle ablation occurred on D-6 and D-5 followed by the placement of an intravaginal progesterone releasing device (CIDR). On D0, the CIDR was removed and ULO (OVEX) was performed by excision of the ovary containing the largest follicle. Prior to ULO on D0 and after ULO, heifers were evaluated by transrectal ovarian ultrasonography to determine AFC and blood samples were taken to determine AMH as shown in the figure.

Ultrasonography evaluations

Ovarian transrectal B-mode ultrasonography was performed using a 7.5 MHz linear-array transducer (MyLabFive, Esaote, Canadian Veterinary Imaging, Georgetown, Ontario, Canada). At each examination, the AFC was determined by counting all follicles > 1 mm in ultrasonography recordings. Antral follicle counts were determined immediately prior to ULO on D0, where the follicle counts of both ovaries were combined to establish the pre-ULO baseline for each animal. Following ULO, ultrasounds were performed on the remaining ovary daily for the first 9 days (D1 to D9), followed by evaluations every ~4 days (D13, D17 and D22), then weekly between D29 and D92, and approximately biweekly thereafter until D230.

Quantification of anti-Müllerian hormone

Blood samples were collected by coccygeal venipuncture into evacuated serum tubes prior to ULO on D0. After ULO, additional samples were collected on D1 - D3, D5, and D7, then weekly

from D14 – D92, then approximately biweekly until D230. All blood samples were centrifuged at 1300 g for 20 minutes, and the serum was transferred into vials and stored at -20ºC until assayed. AMH was determined by enzyme-linked immunosorbent assay (ELISA) using a commercially available bovine AMH ELISA kit (#AL-114, Anshlabs, Webster, TX,) following manufacturers guidelines. Briefly, 50 μL of serum were added to AMH antibody coated wells and incubated for 120 minutes. A second incubation was performed with 100 μL biotinylated AMH antibody solution for 60 minutes at room temperature. The wells were subsequently incubated with streptavidin horseradish peroxidase conjugate solution for 30 minutes. The TMB chromogen solution (100 μL) was added to each well and plates were incubated for 10 - 12 minutes and color development monitored visually. Acid stopping solution (100 μL) was added to each well to stop the reaction. The absorbance of each well was determined at 450 nm using a plate reader (SpectraMax 384, Molecular Devices). Results were obtained using a cubic regression curve-fit as indicated by the manufacturer.

Data arrangement and statistical analysis

The pre-ULO AFC and AMH obtained on D0 were used as the baseline for each individual heifer. Subsequently, AFC and AMH values obtained after ULO were expressed as a percent change from D0 for each heifer. Ovulation rate was determined using the number of corpus lutea, indicative of the number of follicles that ovulated, present at each ultrasound evaluation. All statistical analyses were performed using SAS software, version 9.4 (SAS Institute Inc., Cary, NC). The analysis of variables measured over time (e.g. AFC, ovulation rate and AMH) was performed by analysis of variance using the repeated option in PROC MIXED with day as the repeated variable and cow as the subject with an ante-dependence covariance structure. Genotype, day, and genotype by day interaction were included in the model as fixed effects. The main effects of genotype, day, and their interaction were determined, and pre-planned comparisons between genotypes for specific time points were done by least square difference. A significant difference between treatment groups was considered when $P \leq 0.05$, whereas differences between $P > 0.05$ and $P \leq 0.10$ were considered a tendency. Data are presented as means ± standard error of the mean, obtained using PROC MEANS of SAS.

RESULTS

Effect of ULO on ovulation rate

Ovulation rate of Trio carrier and non-carrier heifers before and after ULO are shown in Figure 2. Prior to ULO, the ovulation rate was greater ($P < 0.01$) in Trio carriers (3.2 ± 0.1) than non-carriers (1.2 ± 0.10). There was an effect of genotype ($P = 0.29$) with greater number of ovulations in Trio carriers compared to non-carriers, however, there was no day ($P = 0.17$) nor genotype by day interaction ($P = 0.29$), indicating that ULO did not affect the ovulation rate of either Trio carriers or non-carriers.

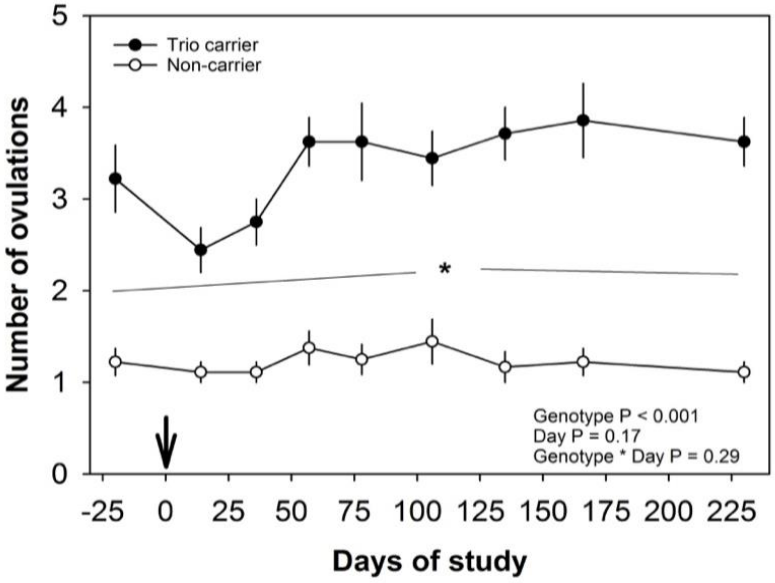

Figure 2. Ovulation rate in Trio carrier and non-carrier heifers prior to and after ULO. The arrow indicates when ULO was performed on D0. *Indicates differences ($P < 0.05$) between genotypes at each timepoint.

Antral Follicle Count

Antral follicle count and percent change of AFC are shown in Figure 3. Prior to ULO, AFC was not different ($P > 0.7$) between Trio carrier (21.2 ± 1.9 follicles) and non-carrier heifers (22.1 ± 1.4 follicles). There was an effect of genotype ($P < 0.001$), day ($P < 0.001$) and a tendency ($P < 0.07$) for a genotype by day interaction on AFC (Figure 3A). By D1 after ULO, the average number of antral follicles in Trio carriers was reduced from 21.2 ± 1.9 follicles between the two ovaries prior to ULO to 10.2 ± 0.9 (49.7%) follicles in the remaining ovary after ULO. Similarly, the AFC of non-carriers decreased from 22.1 ± 1.4 follicles to 10.9 ± 0.6 follicles (50.6%). Mean AFC was greater ($P \leq 0.05$) in Trio carrier than non-carrier heifers on days 9, 14, 106, 121 and 135 after ULO. There was an effect of genotype ($P < 0.001$) and day ($P < 0.001$) on the percent change in AFC, however, no genotype by day interaction ($P = 0.16$; Figure 3B). Based on the percent change model, from D85 to the conclusion of the study at D230, Trio carriers had a greater percent AFC ($P < 0.05$) than non-carrier heifers. Ovarian compensation based on AFC was defined for each individual heifer as the percent change of AFC reaching 100% or more after ULO. Based on percent change AFC, ovarian compensation occurred in 77.8% (7/9) of heifers of both genotypes ($P = 0.99$), however, in the heifers in which compensation occurred, this was observed earlier in Trio carrier (65.8 ± 14 days) than non-carrier (131 ± 29 days) heifers ($P = 0.05$). At the end of the study (D230), average percent change AFC was 119% and 91% of pre-ULO levels in Trio carrier and non-carrier heifers, respectively.

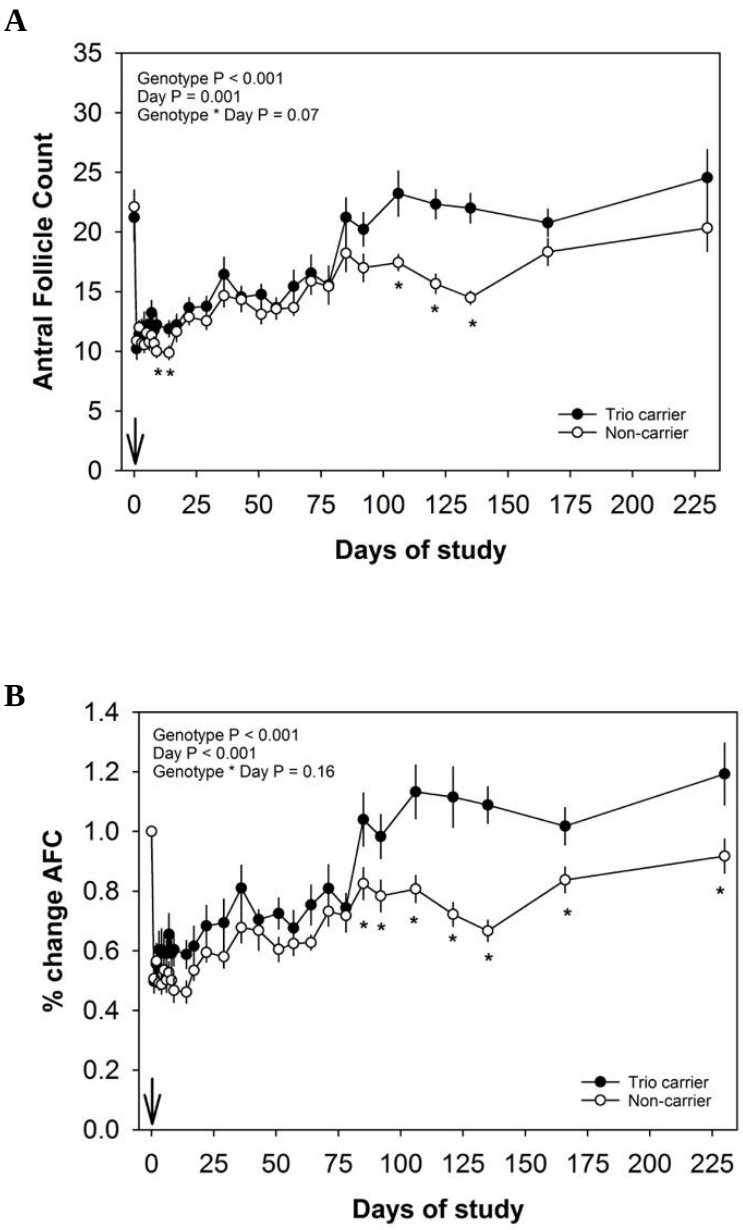

Figure 3. Average antral follicle count (A) and percent change in antral follicle count from pre-ULO (B) in heifers with or without the Trio allele. *Indicates differences ($P < 0.05$) between genotypes at a specific timepoint.

Anti-Müllerian Hormone

Circulating AMH and percent change of AMH are shown in Figure 4. Prior to ULO on D0, AMH concentration was not different ($P > 0.7$) in Trio carriers (349.7 ± 51 pg/ml) and non-carriers (334.8 ± 49 pg/ml). There was an effect of genotype ($P < 0.001$), however, no day ($P = 0.13$) nor genotype by day interaction ($P = 0.29$) on circulating AMH (Figure 4A). Circulating AMH in Trio carriers decreased from 349.7 ± 51 pg/ml prior to ULO to 208.9 ± 37 pg/ml (57.4%) by D3 after ULO. Similarly, AMH in non-carriers was reduced from 334.8 ± 49 pg/ml prior to ULO to 160.6 ± 23 pg/ml (48.8%) by D3 after ULO. Mean circulating AMH was greater ($P \leq 0.05$) in Trio carrier than non-carrier heifers on days 121, 166, and 230 after ULO. There was an effect ($P < 0.001$) of genotype, day ($P < 0.001$), and genotype by day ($P < 0.001$) interaction on the percent change of circulating AMH (Figure 4B). Based on percent change, Trio carriers had greater ($P < 0.05$) percent AMH on days 22, 71, 78, and 106 until the end of the study (D230). Ovarian compensation based on AMH was defined for each individual heifer as the return of AMH serum concentrations to 100% or greater. Based on percent change AMH, ovarian compensation occurred in 33.3% (3/9) of Trio carrier heifers, at an average of 188.7 ± 41 days, while ovarian compensation was observed in 0% (0/9) of non-carrier heifers by the end of the study ($P = 0.21$). At the end of the study (D230), average percent AMH change was 94.6% and 48.1% of pre-ULO values in Trio carrier and non-carrier heifers, respectively.

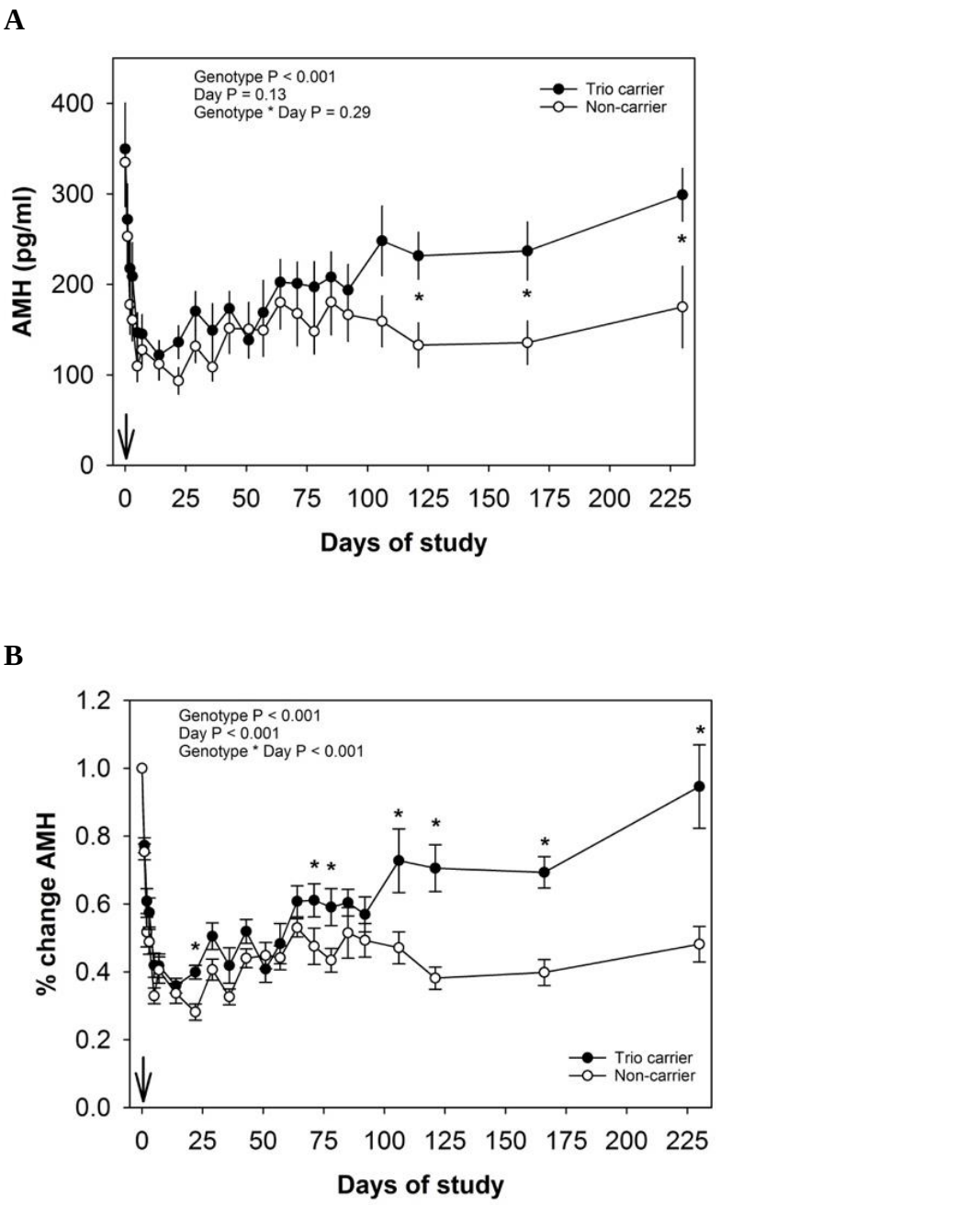

Figure 4. Average circulating AMH (A) and percent change in circulating AMH from pre-ULO (B) in heifers with or without the Trio allele. *Indicates differences ($P < 0.05$) between genotypes at a specific timepoint.

DISCUSSION

Significant advances in the understanding of the role of the TGF-β superfamily of factors in folliculogenesis and the control of ovulation rate in ruminants has been due to the discovery of high fecundity genotypes in both sheep and cattle [8]. Furthermore, the recent discovery of the Trio allele in cattle which leads to multiple ovulations has provided evidence for the involvement of SMAD6, an intracellular mediator of TGF-β signaling in the final stages of follicle development. There is, however, a lack of information on the role of SMAD6 during early folliculogenesis. Ovarian compensatory hypertrophy after ULO has been described in multiple species, however, results from studies in cattle are inconsistent. Because ovarian compensation after ULO would require increased activation of follicles from the primordial pool, the time required for compensation to occur would provide a precise estimate of the length of time required for folliculogenesis to be completed. The present study utilized a ULO model and the unique combination of transrectal ultrasonography and circulating AMH quantitation to determine the presence of ovarian compensatory hypertrophy, the length of folliculogenesis, and the effect of the Trio allele during early folliculogenesis. In addition, the effect of ULO on ovulation rate was evaluated in both Trio carrier and non-carrier control heifers. Results obtained from the present study indicate that ovulation rate remains unaltered after ULO, with Trio carriers having greater number of ovulations as previously described [9, 13]. Ovarian compensatory hypertrophy after ULO was evidenced, albeit earlier in Trio carriers than non-carriers. More interestingly, the definition used to determine ovarian compensation based on the percent change of AFC or AMH from the pre-ULO baseline indicated differences in the percentage of heifers in which ovarian compensation occurred and the timing of such compensation. Ovarian compensation was observed when evaluating AFC in both Trio carrier and non-carrier heifers to a similar extent, however, based on AMH ovarian compensation was observed in ~30% of Trio carrier and 0% of non-carrier heifers. These results taken together indicate the possibility of an uncoupling of the AFC - AMH relationship after ULO.

The ovulation rate of heifers heterozygous for the Trio allele averaged 3.2 ovulations per cycle, which was significantly greater than the ovulation rate of non-carrier control heifers which averaged 1.2 ovulations per cycle, consistent with previous reports on the effect of the Trio allele on ovulation rate [9]. We hypothesized that ovulation rate after ULO would remain unchanged in each genotype due to the presence of the follicle selection mechanism. Our hypothesis was

supported by the observation that ovulation rate was not different before or after ULO in neither Trio carrier nor non-carrier heifers, indicating that ULO did not have an effect on ovulation rate. As a result, ovulation rate continued to be greater in Trio carriers than non-carriers after ULO. The results presented herein are in agreement with a previous report in beef cows, where ULO did not affect the number of ovulations in the first cycle after surgery [11]. Conversely, one study performed on prepubertal heifers reported an increase in ovulation rate for up to three estrous cycles after ULO, doubling the number of co-dominant follicles and increasing the likelihood for twinning [10]. Additionally, in sheep, an increase in ovulation rate after ULO was described at the end of the first cycle following ULO but was not different than control sheep two months later [2]. Conversely, results from another study in ewes indicated that ULO did not result in changes in ovulation rates [23]. The precise reasons for the observed differences between studies in both cattle and sheep remain to be ascertain, however, one potential mechanism is the precise ovary being removed in relation to the presence of the corpus luteum (CL) responsible for progesterone (P4) production. If the ovary being removed were the one containing the CL, then circulating P4 would be significantly reduced which can in turn increase the number of ovulations as previously reported [24]. In the present study, a CIDR device was administered following ULO in order to provide a similar P4 environment to both genotypes, therefore, preventing the increase in ovulation rate observed in other studies.

Unilateral ovariectomy results in ovarian compensatory hypertrophy in swine, however, results from studies in cattle are conflicting. One of the challenges for the identification of ovarian compensation stems from how compensation is defined. Differing methods have been utilized in previous studies such as total ovarian weight, follicular fluid weight, and follicle numbers at different stages of folliculogenesis. Regardless of the method used ovarian compensation is typically defined as the observation of, for example, a number of antral follicles in the retained ovary, after ULO, that is equivalent to the number of antral follicles for both ovaries combined prior to ULO. We hypothesized that removal of one ovary would result in a reduction of ~50% in either AFC or AMH immediately after ULO, and that ovarian compensation by the retained ovary would occur. Our hypothesis was supported at least in part based on the following observations: 1) there was a reduction of AFC and AMH to ~50% of pre-ULO levels by 24 to 72 h respectively; and 2) AFC returned to pre-ULO values in nearly 80% of the heifers of both genotypes by the end of the study. These results are in agreement with those obtained in two previous studies in cattle

[3,4]. For example, Lammoglia et al. [3] described a compensatory mechanism after ULO based on the total number of follicles, determined by transrectal ultrasonography, in each follicular wave during the second estrous cyle after ULO. In addition, another study in prepuberal heifers suggested ovarian compensation occuring based on ovarian and follicular fluid weight suggesting compensation based on short term changes in FSH secretion [4]. In contrast, two other studies in cattle found no evidence in ovarian compensation based on follicle numbers at 6 or > 60 days after ULO [5, 7]. Similarly, a study in ewes indicated that small and medium size follicle number on a per ovary basis did not differ between ULO or control ewes during the first cycle after surgery and 2 months later, however, the number of large sized follicles was greater in ULO ewes [2]. The authors concluded that ovarian compensation based on follicle number did not occur, however, the number of total follicles per ovary was not reported. It is clear that research on the effects of ULO on ovarian compensatory hypertrophy have provided conflicting results and the reasons behind such differences remain unknown. The underlying causes for the contradicting results may be due to variations in the time of ULO, measurements taken and/or the length of time after ULO during which evaluation were undertaken. Based on the present results, ovarian compensation appears to occur in cattle after ULO, at least based on AFC, however, the design of this experiment probably did not provide sufficient time for compensation to occur in all animals.

One of the unique and novel approaches utilized in this study was the utilization of circulating AMH to evaluate ovarian compensatory hypertrophy. Anti-Müllerian hormone is a member of the TGF-β family specifically expressed in the gonads. In females, AMH production is restricted to granulosa cells of growing follicles with AMH concentrations being greatest in large preantral and small antral follicles and decreasing in the later stages of folliculogenesis, thus, circulating AMH concentrations have been used as an indicator of the total number of follicles in an individual [20]. Circulating concentrations of AMH are positively correlated ($r = 0.59$ to 0.88) with the AFC as indicated by results from multiple studies in cattle [20, 25]. From a physiological perspective, AMH has been implicated as an important inhibitor of the primordial to preantral follicle transitional development in multiple species [20, 26]. Based on our hypothesis, we anticipated that circulating AMH would decrease shortly after ULO to ~50%, removing the inhibitory effect on the primordial follicle activation to then return to pre-ULO levels due to increased number of preantral follicles entering the growing pool. Circulating AMH was reduced to ~ 50% by 72 h after ULO, however, ovarian compensation was only observed in 33% of Trio

carrier heifers, while in none of the non-carrier heifers. The correlation of AFC and circulating AMH has been well documented, thus the differences observed in ovarian compensation based on AMH and AFC are intriguing. The results obtained herein suggest an uncoupling of the AFC - AMH association through an undefined mechanism that should be the focus of future research. Ovarian compensation based on AFC was identified at 66 and 131 days after ULO in Trio carrier and non-carrier heifers, respectively, while ovarian compensation based on AMH in Trio carrier heifers was observed at 188 days. As a result, if the difference observed in timing of ovarian compensation between Trio carrier and non-carrier heifers were to be mantained for AMH, ovarian compensation in non-carrier heifers would be expected at ~254 days. Thus, it is plausible that the lack of compensation, based on AMH, in non-carrier heifers may due to insufficient time for proper monitoring in the present study.

One of the premises of the present study was that if ovarian compensation was to be observed, this would allow us to gain knowledge on the length of folliculogenesis. This premise assumed that, in order for ovarian compensation to happen, primordial follicle activation would have to be increased and activated follicles would transition the different developmental stages until the antral follicle population, thus an increase in AFC/AMH would be observed. The precise amount of time required from the time a primordial follicle is activated until it reaches the ovulatory stage is uncertain. Lussier et al. [27], have provided to date an estimation based on a detailed histological study focusing on the mitotic index of granulosa cells and the required amount of time for the number of granulosa cells to double in number. Based on the observations reported, the smallest antral follicle (~ 0.13 mm) would require 41.5 days to achieve an 8.6 mm size, while the transition from 0.13 mm to 1.5 mm would require 30.3 days [27]. It is important to note that these calculations involve antral follicle development and do not refer to preantral follicle development. These observations led others to extrapolate the required time to transition preantral developmental stages, arriving at an estimate for the length of the entire process of folliculogenesis of 60 to 80 days [28]. Results obtained in the present study indicate that in wild-type non-carrier cattle, folliculogenesis requires on average of 131 days, although significant variation was evidenced among individuals. As a result, the length of folliculogenesis is significantly longer than previously reported. One limitation of our approach to determine the length of folliculogenesis is that ovarian compensation could not only originate from increased primordial follicle activation,

but rather increased follicle survival in later stages of development. However, given the number of days required in non-carrier cattle to attain ovarian compensation, this seems unlikely.

Trio carriers have a reduced follicular growth rate during the later stages of antral follicle development, allowing the development of multiple dominant ovulatory follicles that are smaller sized in comparison to wild-type non-carrier cattle [16, 17]. Preantral follicles in sheep carrying a high fecundity allele are smaller in size with fewer granulosa cells compared to follicles of wild-type ewes [19]. Both BMP15 and GDF9, produced by the oocyte, are regulators of granulosa cell proliferation in sheep and their biological actions are found to be decreased in sheep carrying high fecundity alleles [12]. Similarly, in Trio carrier cattle SMAD6 mRNA transcript, an inhibitor of BMP15 and GDF9 signaling, is increased and thus the reduced follicle size is likely due to reduced number of granulosa cells [12, 15]. As a result, we hypothesized that the length of folliculogenesis, defined as the number of days required for each genotype to exhibit ovarian compensation, would be prolonged in Trio carriers than non-carriers. Surprisingly, results obtained in the present study indicate that heifers carrying the Trio allele in which ovarian compensation was noticed, based on AFC, required less time than in non-carrier heifers, therefore, theoretically having a faster progression through folliculogenesis. This intriguing observation is further supported by the observations concerning AMH, as only Trio carriers had evidence of ovarian compensation based on AMH, albeit in a small percentage of them. It is unknown at this time the explanation behind the earlier ovarian compensation observed in Trio carriers, however, this could be due to either a faster progression through preantral follicle development or a greater rate of primordial follicle activation. Future research should focus on investigating these two possible mechanisms.

CONCLUSION

Folliculogenesis is a complex process involving multiple steps of unknown duration, and methods available to dynamically monitor it are currently lacking. Trio, a novel high ovulation allele, results in increased expression of SMAD6, which in turn reduces antral follicle growth rate and results in multiple smaller sized ovulatory follicles The present study used a ULO model to investigate the presence of ovarian compensatory hypertrophy, the length of folliculogenesis, and the effect of the Trio allele on early folliculogenesis. Results indicated that: ULO decreased AFC and AMH to ~50%, ovarian compensation based on AFC was observed at ~2 and ~4 months after ULO in Trio carrier and non-carriers, respectively; ovarian compensation, based on AMH,

occurred in Trio carriers (~8 months) but not in non-carriers by conclusion of the study. Results obtained from this study further the understanding of the process of folliculogenesis and the control of ovulation rate in monovular species.

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